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THE MEASUREMENT OF OESTRONE, EQUILIN AND DEHYDROEPIANDROSTERONE IN THE PERIPHERAL PLASMA OF PREGNANT PONY MARES BY RADIOIMMUNOASSAY

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SUMMARY

Oestrone, equilin and dehydroepiandrosterone were measured by radioimmunoassay in peripheral plasma from five pony mares; samples were taken from day 60 of pregnancy at approximately weekly intervals through to parturition. Of the three steroids measured, dehydroepiandrosterone showed the most consistent pattern of secretion. DHA concentrations remained low until day 105, increased rapidly from day 120 and attained maximum values between days 168–210; levels declined to day 300, but there was considerable variation between mares in DHA patterns prior to parturition. There was great variation between mares in the relative concentrations of oestrone and equilin. There was no obvious relationship between the elevated concentrations of dehydroepiandrosterone observed and the pattern of secretion of either oestrogen.

INTRODUCTION

The two major equine pregnancy oestrogens, oestrone and equilin (3-hydroxy-1,3,5(10),7-estratetraen-17-one), have been found to reach their maximum concentrations in plasma and urine between days 150–179 and days 210–239 of gestation respectively [1, 2]. This pattern of total oestrogen excretion is paralleled by the growth and subsequent regression of the equine foetal gonads [3, 4] which have been implicated as a source of oestrogen or oestrogen precursors [5]. The foetal gonads contain large amounts of dehydroepiandrosterone (DHA) [6] which has been shown to be a precursor of oestrone [7], but the status of DHA as a precursor of equilin is a matter of dispute [7,8]. In order to investigate any relationship between DHA and equine oestrogen production, the concentrations of oestrone, equilin and DHA were determined in jugular vein plasma taken from five mares from day 60 of pregnancy to term.

MATERIALS AND METHODS

Plasma samples were taken by Mr R. Parkes, MRCVS, of the Equine Research Station, Balaton Lodge, Newmarket. The mares were bled in the morning at approximately weekly intervals throughout pregnancy. One mare, Dumpling, showed a vaginal discharge at the beginning of the fifth month of pregnancy. At term, her foal was weak and undersized. Plasma samples were stored at −20°C until assay.

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[7-^{3}H]Dehydroepiandrosterone (S.A. 16.6 Ci/mmol), were obtained from the Radiochemical Centre, Amersham. Equilin was a gift from Ayerst, McKenna and Harison (Montreal, Canada) and all other steroids were obtained from Steraloids Inc. (Wilton, U.S.A.). *Helix pomatia* was obtained from I.B.F. (Paris, France). General reagents were of Analar grade from B.D.H. (Poole, Dorset); all solvents were redistilled before use.

Phosphate buffered saline with thiomersal (PBSM) consisted of 0.1 M phosphate buffer pH 7.0 containing sodium chloride (0.15 M), thiomersal (0.01% w/v) and Knox gel (0.1%) and was used for all dilutions. Charcoal (0.25 g; Norit A Sigma) was suspended in PBSM (100 ml) containing Dextran T-40 (2.5 mg, Pharmacia). Liquid Scintillation counting was done in toluene (5 ml) containing 2,5-diphenyloxazole (PPO, 4 g/l) and 1,4-bis [2(5-phenyloxazolyl)] benzene (POPOP, 0.1 g/l) using a Packard Tri-carb liquid scintillation spectrometer Model 2450.

The preparation and characterisation of antisera specific for oestrone, equilin and DHA have been described previously [9, 10, 11].

Radioimmunoassay of oestrone. The major fraction of oestrogens present in equine pregnancy plasma and urine are conjugated [7], so it was necessary to hydrolyse the plasma samples prior to oestrogen analysis using *Helix pomatia* enzyme [12]. Furthermore, due to the high concentration of oestrone found in equine pregnancy plasma [1] a dilution step was essential to permit the measurement of the samples within the range of the standard curve. The oestrone assay will be described in detail. Either 50 μl or 100 μl plasma samples (dependent upon the stage of pregnancy) in duplicate were pipetted into stoppered tubes and in-

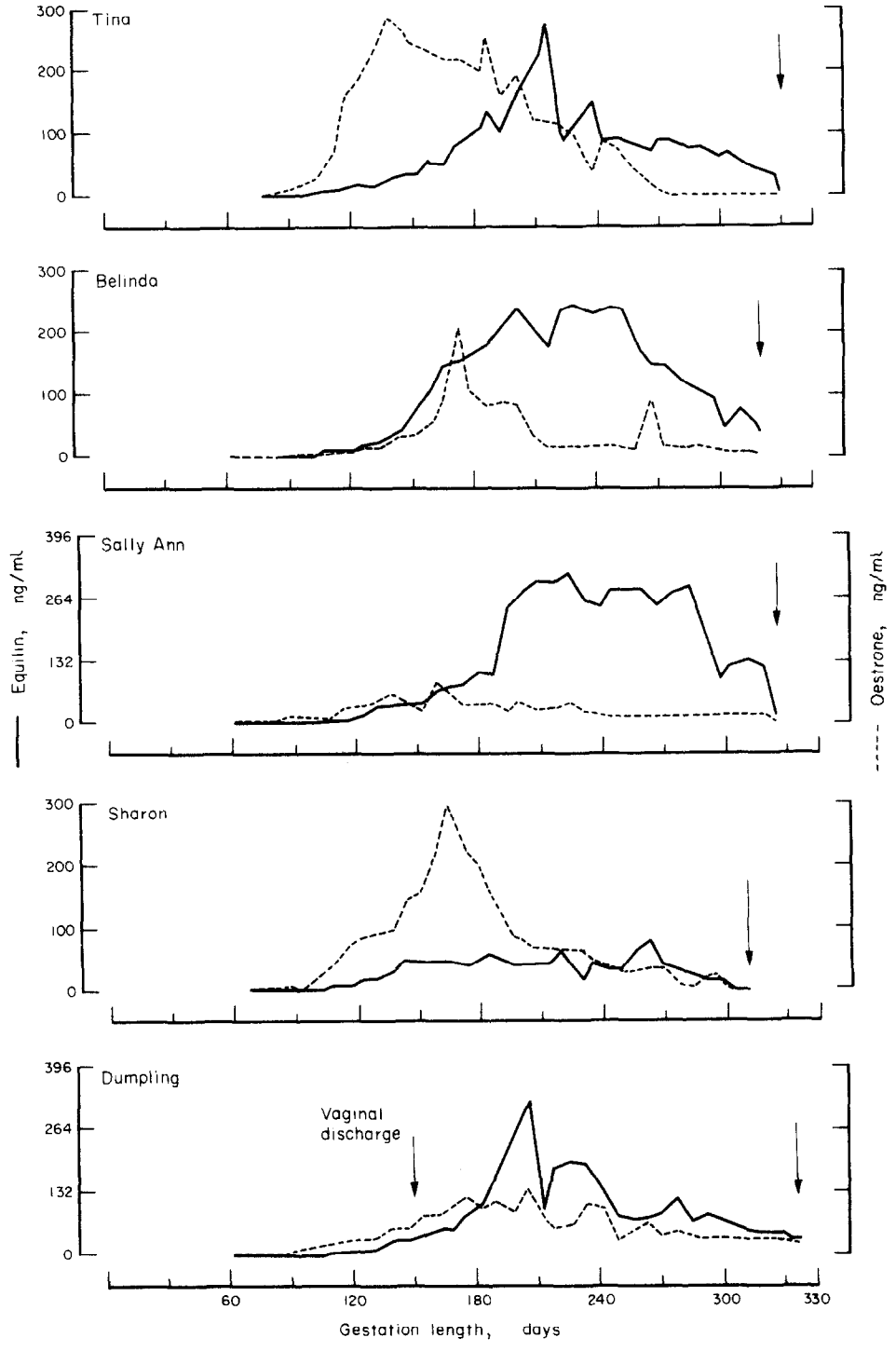

Fig. 1. Equilin and oestrone concentrations in the peripheral plasma of five pregnant mares throughout gestation. Arrow indicates day of parturition.

cubated with *Helix pomatia* enzyme (10 μl) for 30 min at 56°C to hydrolyse any conjugates present. The samples were cooled and vortexed for 10 s with 3 ml diethyl ether. The tubes were placed in an acetone–solid carbon dioxide mixture and the ether layer was decanted into 12 × 75 mm tubes. 110 μl aliquots of PBSM gel were similarly extracted to provide an estimate of non specific interference.

A stock ethanolic solution of oestrone (1 pg/μl) was used to prepare a standard curve ranging from 0–250 pg. Solvents were evaporated under nitrogen at 37°C. The dried extracts were redissolved with vortexing in 500 μl or 1 ml PBSM gel according to their anticipated concentration. A 10 μl aliquot was taken from each diluted extract and transferred to a second assay tube.

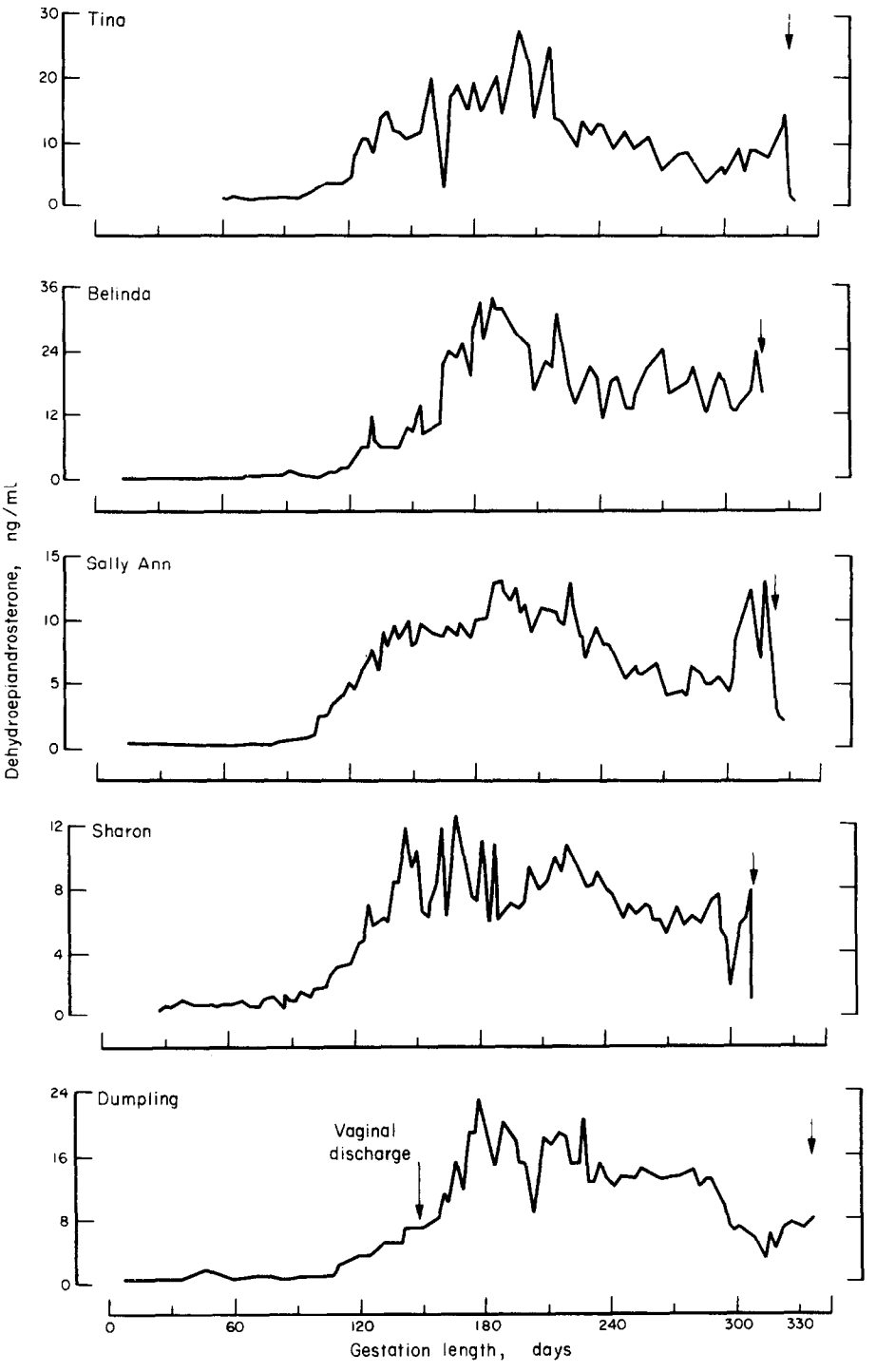

Fig. 2. Dehydroepiandrosterone concentrations in the peripheral plasma of five pregnant mares throughout gestation. Arrow indicates day of parturition.

Equal volumes of oestrone antibody (1:3,000 dilution) and labelled oestrone (diluted to 12,000 d.p.m./100 μl with PBSM) were mixed and 200 μl added to each assay tube. To standardise the assay volume 10 μl PBSM was added to the standard curve tubes to give a total volume of 210 μl per tube. The tubes were vortexed and left to equilibrate overnight at 4°C.

The final stages of the assay were carried out at 4°C. Charcoal suspension (0.5 ml) was added to each tube. The tubes were vortexed and allowed to stand for 10 min. They were centrifuged for 10 min at 2,500 *g*, the supernatants were decanted into scintillation vials and counted.

Radioimmunoassay of DHA. Plasma samples were analysed without hydrolysis, as described previously

[11] although smaller plasma volumes (10 and 20 μl) were extracted.

Radioimmunoassay of Equilin. This assay, employing an equilin 17-(O-carboxymethyl) oxime [^{125}I]-iodohistamine conjugate as tracer was performed as described previously [10].

RESULTS

The results obtained from the measurement of equilin, oestrone and DHA in the peripheral plasma of the five mares are shown in Fig. 1 and 2. The assay characteristics of the equilin radioimmunoassay were as follows: the standard curve was linear, on a semi log dose–response plot from 0.5 ng to 20.0 ng; the least detectable quantity distinguishable from zero was 0.5 ng ($P < 0.01$). The intra-assay coefficient of variation was 5.5% at 2.4 ng and 6.5% at 16.0 ng ($n = 8$). The inter-assay coefficients for the same controls were 9.2% and 13.1% ($n = 6$) respectively. The regression coefficient b, was 0.97. The most relevant cross-reactions are oestrone (7.3%), equilenin (0.7%) and DHA (0.1%) [10]. Plasma blanks (200 μl) taken from a non-pregnant mare were below the limit of sensitivity, as were the PBSM blanks.

The DHA antibody gave a standard curve which was linear, on a semi log dose-response plot from 10 pg to 400 pg and the least detectable quantity of DHA was found to be 5 pg ($P < 0.01$). The intra-assay coefficient of variation at 10 pg was 3.2% and at 200 pg was 6.9% ($n = 6$), the inter-assay coefficients of these controls were 7.4% and 8.1% ($n = 12$) respectively. The cross-reaction were oestrone (0.68%), equilin (0.07%), equilenin (0.04%) and 7α-hydroxydehydroepiandrosterone (0.04%); PBSM blanks were 10 pg/200 μl.

The oestrone antibody gave a standard curve which was linear between 20 pg and 250 pg on a semi log plot with a sensitivity of 10 pg ($P < 0.01$). The intra-assay coefficient of variation at 30 pg was 3.0% and at 90 pg was 2.2% ($n = 8$), the interassay coefficients of variation for the same controls were 8.8% and 9.3% respectively. The regression coefficient b, was 0.98. The cross-reactions were equilin (3%), equilenin (7.2%) and DHA (0.1%), PBSM blanks were 20 pg/200 μl.

DISCUSSION

We have measured the peripheral plasma concentrations of DHA, equilin and oestrone in the pregnant mare, at regular intervals, from day 60 through to parturition. The overall pattern of secretion of the two oestrogens was similar to that obtained by Cox [1] who used a gas–liquid chromatographic method. DHA concentrations in the pregnant mare have not been measured previously. We found that the concentrations of DHA increase markedly during pregnancy and may be up to 1000 times greater than the concentrations measured in the non-pregnant mare [11].

Of the three steroids measured, DHA showed the most consistent pattern of secretion in the five mares. In all mares DHA concentrations rose above 1 ng/ml by day 105, increased rapidly from day 120 to maximum values of 12–34 ng/ml between days 168–210, followed by a general decline to day 300. However, there was a considerable variation between mares prior to birth. In two mares, Sally Ann and Belinda, concentrations were slightly increased on the fifth day before parturition. In Tina, an increase in DHA concentration was detected on the day before birth, but there was no obvious change in the previous two mares, possibly due to infrequent sampling. Nevertheless, there was a fall in DHA concentrations from 16.75–2.8 ng/ml to between 0.16 and 2.4 ng/ml within 24 h of parturition in all four mares.

The sustained high concentrations of DHA throughout most of pregnancy taken with the almost immediate return to normal concentrations following parturition indicate that the foetal–placental unit is the source of the extra DHA. Placental DHA has not been determined but enzyme determinations have established the presence of 5ene-3β-hydroxysteroid dehydrogenase in the placenta and foetal adrenal cortex between days 122–210, indicating that these are unsuitable sites for DHA production [13].

Only traces of this enzyme have been detected in the foetal gonads [13]. Ultra-structural studies [14, 15] indicate that the foetal gondad is capable of steroid biosynthesis and Raeside [6] has shown that DHA is in fact the principal product of the foetal gonad. Furthermore, the pattern of DHA secretion we have observed is remarkably similar to the pattern of growth and subsequent regression of the foetal gonad which begins to enlarge during the fourth month of pregnancy, continues to grow during the following 3–4 months and then begins to regress during the eigth month [3, 4]. So for the present, it must be assumed that the foetal gonads are the major source of the DHA in the peripheral plasma of the pregnant mare.

The concentrations of DHA show considerable variation within a few days, indicating either pulsatile release or an erratic production of the steroid.

The pattern and quantity of oestrogen secretion varied greatly between mares. Total oestrone rose above 1 ng/ml by day 90, attaining maximum values of 92–300 ng/ml between days 138–142 in four mares. Equilin was detectable in plasma by day 108 in all mares and started to rise in concentration by about day 150. Maximum values of 79–340 ng/ml for equilin were obtained between days 214–260 in four mares. The fifth mare, Dumpling, had a vaginal discharge at about day 150 and had both equilin and oestrone maxima on day 204. In all mares oestrogen concentrations declined to term, although oestrone concentrations (1–5 ng/ml) fell further than equilin concentrations (6–43 ng/ml). Both oestrogens declined to very low concentrations within 24 h following parturition.

The general pattern of oestrogen secretion described by Cox employing a gas–liquid chromatographic method, is in good agreement with the present study although the concentrations of both equilin and oestrone recorded here are higher than recorded by Cox. Cox found that equilin concentrations were greatly reduced from day 304 to term and we also have shown a progressive decline at this time. However, Cox found that oestrone concentrations were higher than equilin concentrations from day 305 to term. In all five mares we found the reverse trend, which is in accordance with the observations of Barnes *et al.* [16]. No explanation can be offered for the different pattern of equilin and oestrone secretion prior to parturition. The consistently higher concentrations of equilin found here may be due to the use of enzyme hydrolysis as opposed to the hot acid hydrolysis employed by Cox which may have partially destroyed equilin.

In vivo studies [7] have shown that DHA is a precursor of oestrone but not of equilin, although *in vitro* work [8] has shown that the equine placenta is capable of transforming DHA into equilin. No conclusions can be drawn from the present work concerning the biogenesis of equilin. The prolonged period of high DHA secretion encompasses the period of maximum production of both oestrone and equilin, so any causal relationship which might exist between these steroids is masked. However, there remains the mystery of why oestrone concentrations should rise and fall whilst an oestrone precursor, DHA, is maintained at elevated concentrations. The sudden increase in oestrone concentration between the third and fourth month of gestation may be due to the production of DHA from the hypertrophied foetal gonads and to the maturation of the aromatising enzymes of the established placenta. Equilin biosynthesis must also require such aromatising enzymes, so it cannot be the breakdown of these enzymes which causes the decline of oestrone concentrations, for equilin attains maximum concentrations at this time. The total oestrogen content of plasma from the umbilical artery and vein have shown foetal blood to contain much higher quantities of oestrogen than were in the uterine circulation as late as day 315, indicating the viability of the foetoplacental aromatising enzyme system at that stage [16].

In conclusion, despite the measurement of the three steroids reported the relationship between them remains unresolved.

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